Modeling Particulate Matter Exposure Using Mesh Nebulizers with Human Lung Cells

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Senior Honors Thesis
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April 28, 2025

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Abstract

Particulate matter (PM) is a common air pollutant known to have negative health effects in humans. Investigating the effects and complexities of PM is important; however, concerns exist regarding the biological relevance of one of the most common exposure methods. In a cell culture model, liquid application involves the direct application of liquid condensates to the cell surface. This method has limitations, as it creates a hypoxic environment for cells and has been shown in previous research to cause unintended biological artifacts in airway cells. It is of interest to explore more biologically representative methods of PM exposure. We hypothesize that mesh nebulizers could be an effective alternative for modeling PM exposure in a human lung cell system. Mesh nebulizers turn liquid condensates into a fine aerosol and could potentially be used to deposit PM solutions onto cultured cells. To compare exposure methods, primary human bronchial epithelial cells (HBECs) were exposed to PM using both nebulization and liquid application. Endpoints for inflammation and hypoxia were then assessed to evaluate and compare the two methods. Hypoxia endpoints did not show that liquid application caused significantly higher levels of hypoxia markers compared to controls and nebulizer exposure. Evaluation of inflammatory markers revealed that neither method produced a significant inflammatory dose-response effect, and the data suggested that nebulizers do not induce as consistent an inflammatory response to PM exposure as liquid application. The results of this project do not support the original hypotheses; however, concerns still exist with liquid application, and we believe that mesh nebulizers may be a valuable tool for inhalation toxicology research. These findings suggest further investigation into the effects of liquid application on cultured airway cells, and into nebulizers as an alternative exposure method are needed.

1. Introduction

Particulate Matter (PM) is a form of air pollution originating from both human activities and natural sources, such as vehicles, wildfires, volcanic eruptions, and industrial processes, among others. Inhalation of PM has been shown to have wide-ranging negative effects on human respiratory health and other organ systems. These include asthma, chronic obstructive pulmonary disease (COPD), cardiovascular diseases and even cancers. The impacts of air pollution on human health are so pronounced that the World Health Organization has identified air pollution and climate change as the collective number one threat to human health. [1], [2], [3] As humans continue to burn fossil fuels and climate change exacerbates extreme weather events like wildfires and droughts, the implications of PM on human health will be increasingly important.[4] To study these pollutants researchers must have access to representative models for PM exposure.

Currently, inhalation toxicology researchers use *in vivo* or *in vitro* models along with various exposure methods to study pollutants or toxicants. *In vivo* models of animals and humans are important as highly biologically representative models, and have successfully been used to study air pollution exposures including PM. [5] However, the complexity of pollutants, toxicants, and particles that researchers are interested in has been increasing, and *in vivo* models are often too complex, expensive, or unavailable to meet the demands of researchers. *In vitro* models offer a simpler, often more accessible, and economical medium/high throughput model for research. Hence, *in vitro* models will be necessary to study the broad volume of inhalable substances that need to be studied. [6], [7]

The two main cell culture models used for inhalation toxicology research are primary cells from donors and immortalized cell lines. Immortalized cell lines are popular for being easier to obtain and use in research and they tend to have less variation between experiments; however, because they are immortalized or transformed, it is less clear how well they represent airway physiology. Primary cells are taken directly from humans (either donated lung tissue or donors) and provide more certainty that the samples are biologically representative, including variability due to donor demographics, such as age, sex, and pre-existing disease. Primary cells have the downside of being more expensive, more difficult to obtain, and having the aforementioned variation between different donors. [8] To investigate substances, researchers consider the benefits and limitations of the different cell culture models and choose the best model for their specific project.

In addition, current *in vitro* respiratory cell models are more biologically relevant because they can emulate certain characteristics of the human respiratory system. A major development in the field of inhalation toxicology was the development of the air-liquid interface (ALI) method. In the ALI system, respiratory cells are grown on a semi-permeable membrane that is suspended in a larger well. Media is added into the well underneath the filter (basolateral side), and the surface above the filter (apical side) is left open to the air. [8] Culturing cells at ALI recreates the physiological conditions of the respiratory system where the apical side is exposed to inhaled/exhaled air and the basolateral side to underlying tissue and therefore allows cells to be a more representative model.

To study the effects of various pollutants, including PM, on cells in an ALI system the main exposure method is the direct application of liquid condensates to the cell surface or “liquid application.” In this method, the specific test particle is suspended in a liquid and then the solution is applied to the apical surface of the cell culture. Researchers have shown that this method of exposure causes significant changes in cells including changes in gene expression, biological pathways, protein secretions, and even epithelial barrier integrity. [6], [9] These changes reduce the biological relevance of the ALI lung cell model as *in vivo* the physiological conditions would not be exposed to inhaled liquids. There is a need for further investigation into the specific effects of liquid application and for an alternative method of exposing airway cell models to PM that is biologically representative.

We hypothesize that nebulizers may be an effective alternative to liquid application. Nebulizers are a common medical technology typically used to deliver medicine directly to the lungs. They use compressed air, mesh, or high-frequency vibrations to turn an aqueous solution into an aerosol. [10] Nebulizers are small, readily available, and simple to use. To expose cells to PM, particles would be suspended in a liquid solution, which would be injected into a nebulizer. The nebulizer would then turn the solution into an aerosol and deposit the particles onto the apical surface of the cells. This method would deposit less liquid onto the apical surface of the cells, theoretically reducing unintended effects like hypoxia. There is little literature on the use of nebulizers to expose cell models to PM, but researchers have successfully used nebulization to study the effects of diesel PM on human subjects and in mice. [3], [11] These research projects were done *in vivo* but are still an example supporting the use of a nebulizer to disperse a common form of PM. If nebulizers can effectively deposit PM onto cells, then this method could be a valuable tool for researchers.

High quality exposure methods are important for understanding the toxicological properties of substances. A good exposure method should be easily repeatable and be able to produce consistent results in cells. If nebulizers are an effective exposure method, then the exposures will be simple, robust, and cause a consistent response. To investigate the effects of liquid application on cells we will expose primary human bronchial epithelial cells (HBECs) to PM using liquid application and nebulizer application. We will quantify the change in expression of hypoxia related genes HMOX1 and HIF1A to evaluate the unintended effects of liquid application. To test the effectiveness of nebulizers to deliver PM to airway epithelial cells and elicit biological responses, we will analyze exposed cells for markers of inflammation focusing on whether nebulizer exposure causes an inflammatory response.

2. Materials and methods

2.1 HBEC culture: Primary HBECS were purchased from the University of North Carolina at Chapel Hill Marsico Lung Institute Tissue Procurement and Cell Culture Core (UNC MLI TPC). HBECs at passage 3 were seeded onto collagen coated 6.5mm transwell inserts (Costar #3470). HBECs were initially expanded in flasks, then plated on transwells at passage 3. After reaching confluency under submerged conditions, they were brought to ALI and then maintained for approximately one month until they were fully differentiated. [12], [13], [14]

2.2 Nebulizer operation: Micronice mesh nebulizers with 5 micrometer mesh filters were purchased from Tekceleo. The purchase included a control unit, a circuit board, and all necessary wiring, in addition to the mesh nebulizers. Adapters were then 3D printed to allow nebulizers to sit on top of and deposit into a standard twelve-well cell culture plate. Solutions for nebulization were created before the exposure and were mixed via vortexing and sonicating immediately before nebulization. To nebulize solutions the nebulizers were fitted on top of a cell culture plate, and 75µL of the solution were injected onto the membrane of each nebulizer before turning the nebulizer on.

2.3 PM exposure

2.3.1 PM solutions: PM samples for HBEC exposure were obtained as a PM pellet suspended in methanol. The PM was collected on filters in Xinxiang, China on 05/10/2022.[15] The PM was extracted from filters and stored in methanol. The methanol was evaporated off by leaving the tube open in a sealed container with Drierite for approximately 48 hours. Once the methanol had evaporated off and only a dry pellet remained, the pellet was resuspended with phosphate buffered saline (PBS) to a concentration of 10 mg/mL and sonicated and vortexed to mix. Aliquots at 5.0 mg/mL, 2.5 mg/mL, 1.0 mg/mL, 0.5 mg/mL, and 0.1 mg/mL were then created by diluting the stock in PBS. Aliquots were stored at -20 ˚C.

2.3.2 Cell preparation: The day of exposure, HBECs were washed by adding approximately 150 µL PBS to the apical surface of the cells, allowing the solution to rest for 15 minutes, and then aspirating the solution off. The basolateral media was replaced with 1 mL of fresh ALI media. All cell preparation was performed in a biosafety cabinet.

2.3.3 HBEC PM exposure: The exposure conditions for this experiment were: air control (left at ALI), liquid PBS control, liquid 0.1 mg/mL, liquid 0.5 mg/mL, liquid 1.0 mg/mL, nebulize PBS control, nebulize 0.5 mg/mL, nebulize 1.0 mg/mL, nebulize 2.5 mg/mL, and nebulize 5.0mg/mL. For the air control no solution of any kind was added to the cells, cells were left at ALI. For the liquid treatments, including the control, 150 µL of the respective solution was pipetted directly onto the apical surface of the cells. For the nebulizer treatments, including the control, 75 µL of the respective solutions was nebulized onto the apical surface of the cells according to the nebulizer operation directions described above. After all exposures were completed, the cells were returned to the incubator.

HBEC exposures were repeated in two rounds using two different HBEC donors. There was only one well exposed per donor for each treatment condition. All steps of the cell exposures were performed in a biosafety cabinet.

2.3.4 Sample collection: After four hours of incubation, cells were brought back into the biosafety cabinet for sample collection. Three types of samples were collected: basolateral media, apical wash, and cell lysate. To collect each sample, individual 1.5 mL Eppendorf tubes were labeled for each sample of each well. Basolateral media was collected by directly pipetting out the basolateral media from the well. Apical wash involved rinsing the cell surface with PBS and collecting 300 µL of wash solution. Finally, cell lysate was collected by lysing the cells with a beta-mercaptoethanol solution Purelink RNA Isolation Kit (Catalog #: 12183025) and scraping the transwell surface with a flat tipped pipette. After the cells were lysed, the sample was pipetted into a sample tube.

2.4 Sample analysis:

2.4.1 IL6 and IL8 ELISAs:

Interleukin-6 (IL6) quantification was done on basolateral media samples for HBECs. This was done using an R&D Systems DuoSet enzyme-linked immunosorbent assay (ELISA) human IL6 ELISA kit (Catalog number: DY206). We followed the steps detailed in the kit instructions and used a Clariostar plate reader to quantify the optical density.

Interleukin-8 (IL8) quantification was done on basolateral media samples for HBECs. A 1:30 dilution in reagent diluent was used for the basolateral media. The ELISA was done using an R&D Systems DuoSet ELISA human IL8 ELISA kit (Catalog number: DY208). We followed the steps detailed in the kit instructions and used a Clariostar plate reader to quantify the optical density according to the kit instructions.

2.4.2 qPCR:

RNA purification was done using an Invitrogen™ PureLink™ RNA Mini Kit. We followed the steps detailed in the kit to isolate and purify RNA from each of the cell lysate samples collected from the exposures.

cDNA synthesis was done using an Applied Biosystems High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Cat No. 4374966). We followed the steps detailed in the kit to create cDNA for all cell lysate samples.

Finally, qPCR was completed using an Applied Biosystems TaqMan Universal PCR Master Mix, and Taqman Gene Expression assays for HMOX1 and HIF1A, using ActB as an endogenous control for both. The steps of the protocol are briefly outlined below. Dilute cDNA to 8.33 ng/uL. Create a master mix for each gene consisting of 10 parts TaqMan Universal PCR Master Mix to 1 part TaqMan Gene Expression Assay. On a 96-well optical plate add 11 uL of each master mix to a designated well. Add 9uL of sample to each designated well using nuclease free water as a no template control, which makes the total reaction volume 20 uL. Cover and briefly centrifuge the plate. The PCR samples were then processed on a Quant Studio 3 machine.

2.5 Statistical analysis and visualization:

2.5.1 ELISA Analysis and Visualization:

Statistical analysis and visualization were done using GraphPad Prism and Microscoft Excel. Excel was used for data manipulation and organization, and GraphPad was used for analysis and visualization.

Results for IL6 and IL8 ELISAs were graphed on bar plots using GraphPad and the built in One-Way Anova. The one-way Anova was completed with matching between donors, a gaussian distribution of residuals was assumed, and Sidak's test was used to account for multiple comparisons. We used a family-wise alpha of 0.05.

2.5.2 qPCR Analysis:

The qPCR samples were processed using the Δ ΔCt method. Raw Ct information was pulled from the results of the PCR on the QuantStudio 3 machine. To calculate a fold change in gene expression, the Ct value for the housekeeping gene for a sample is subtracted from the Ct value for the gene of interest for a sample, giving the ΔCt value. To calculate the full ΔΔCt, an additional value is calculated and subtracted from all values. We chose the average of the PBS control ΔCt values to be the additional value. To combat batch effects calculations were split between the two rounds of exposures. The average ΔCt value for the first two donors was calculated and subtracted from all ΔCt values of the first two donors. Then the average ΔCt value for the second two donors was calculated and subtracted from all ΔCt values of the second two donors.

3. Results

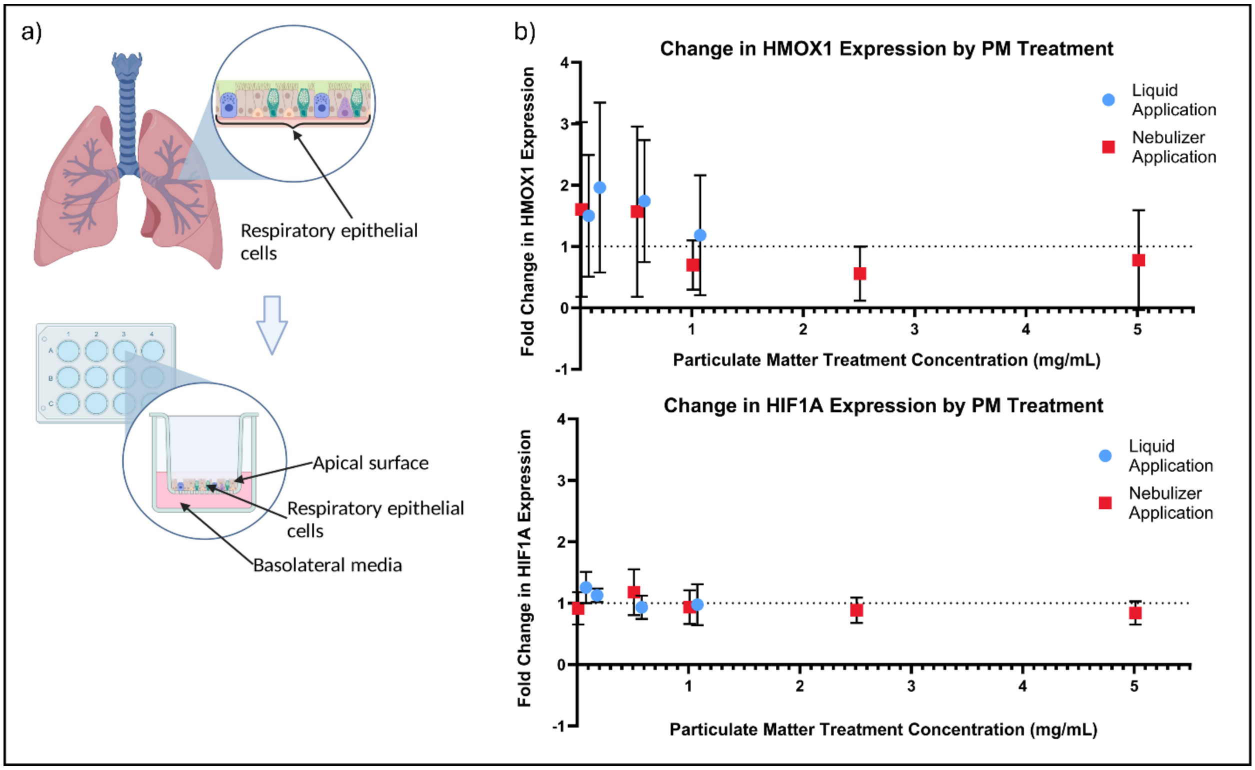

Figure 1: Liquid application of pollutants to respiratory cells cultured at ALI does not increase hypoxia related gene expression. a) Representation of primary human airway cells harvested and then grown on transwells. b) qPCR results for HMOX1 and HIF1A. Fold change in HMOX1 and HIF1A expression is detailed on the vertical axis. The horizontal dotted lines at y = 1 represent the control condition of cells that remained at ALI. At y = 1 there is no change in gene expression. The concentration of PM treatment for nebulizer and liquid application groups is on the x axis.

Liquid exposure did not cause elevated levels of gene expression related to oxidative stress.

Previous research has raised concerns with the method of exposing cultured airway cells to toxicants via liquid exposure. Liquid exposure is suspected to cause unintended hypoxia and changes to biological pathways in cells. Heme oxygenase 1 gene (HMOX1) is gene that indicates inflammation and oxidative stress. Hypoxia-Inducible Factor 1 (HIF-1) is a transcription factor that plays an important role in the physiological response to hypoxia, and HIF-1A is a gene that encodes the alpha subunit of HIF-1. [16] Both of these genes are appropriate to investigate unintended hypoxia caused by liquid exposure. To evaluate the negative effects of liquid exposure and compare it to the proposed alternative, nebulizer exposure, we exposed primary human airway cells using liquid application and nebulizers. Samples of cell lysate were collected, and qPCR was used to quantify HMOX1 and HIF1A expression in samples. There is no clear pattern in the change in gene expression, and we observe large variability in the error bars between and within groups. The results show no significant changes from control across the groups with no significant differences in gene expression change between any groups. The results of this portion of the experiment contradict literature and do not support the hypothesis that liquid application causes elevated levels of hypoxic stress.

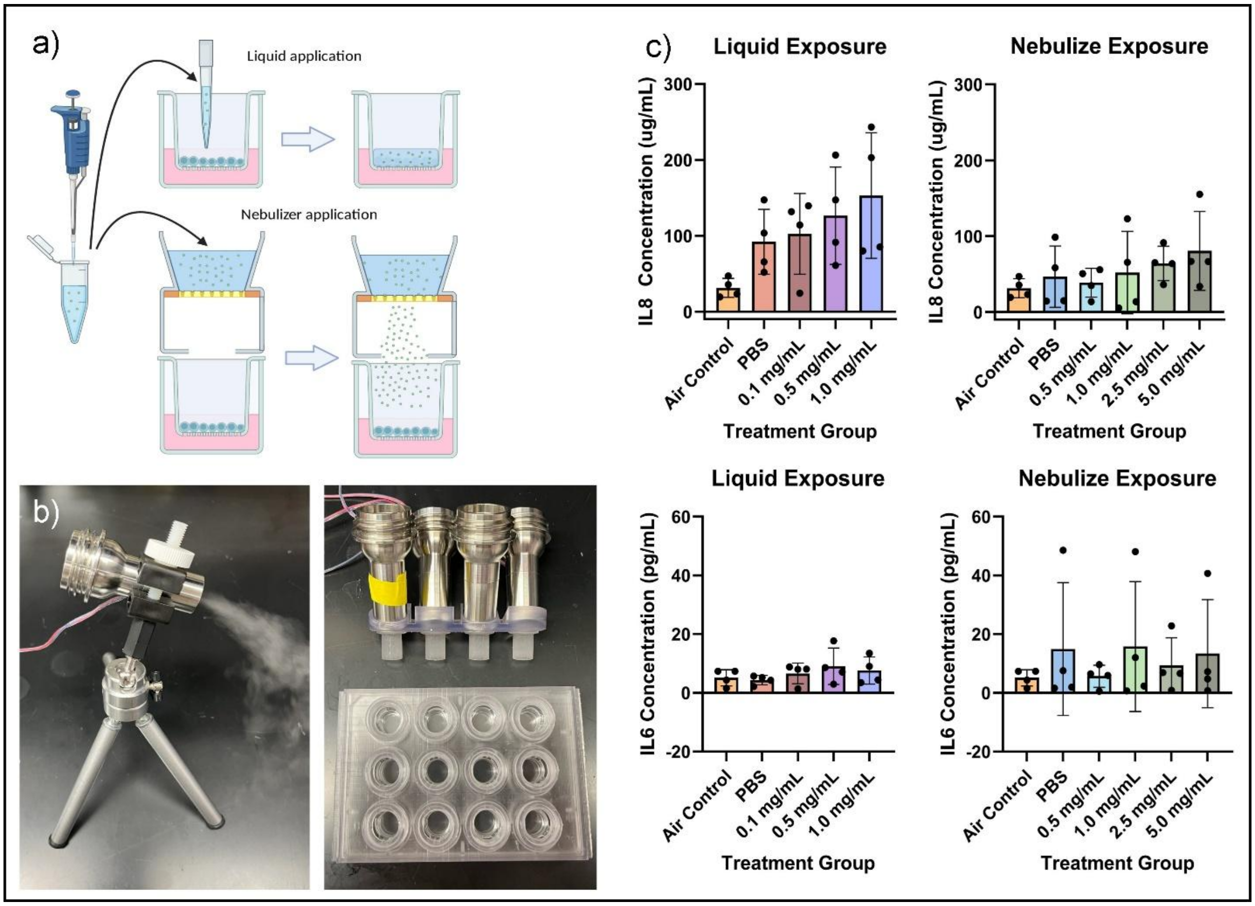

Figure 2: Liquid and nebulizer application of PM pollution did not cause a significant increase in inflammatory cytokine levels. a) Schematics of the difference between liquid application and nebulizer application models. b) Photograph of mesh nebulizer nebulizing PBS (left), and mesh nebulizers seated in adapters for 12 well plate (right). c) ELISA results for Interleukin-8 (top) and Interleukin-6 (bottom). Cytokine concentration is pictured on the vertical axis and cell treatment group on the horizontal axis.

PM exposure did not induce significant inflammatory dose response, and nebulizers did not effectively expose cells.

Nebulizers have previously been used to expose humans and animals to PM; however, there is limited research on the use of nebulizers to expose lung cell models to PM. We hypothesized that mesh nebulizers would be an effective exposure method that also addresses the concerns raised with liquid application. Inflammation is a key endpoint for inhalation toxicology research, so it is an appropriate response to test when evaluating nebulization. To test the effectiveness of mesh nebulizers as an exposure method, we exposed primary human airway cells to PM using both liquid application and nebulizers and then evaluated the inflammatory response of the cells to the exposure. Using statistical tests to evaluate the inflammatory cytokine levels we found that there was no significant difference between any groups for liquid or nebulizer exposure for IL8 and IL6. We can observe a nonsignificant dose response for IL8 levels as shown by the positive trend in the top two graphs of figure 2c, but there is a large amount of variability between donors preventing significant differences and leading to large error bars. This result, particularly the lack of a significant dose response to liquid application, raises concern with limitations of the experimental design and does not provide evidence that nebulizers are an effective exposure method for airway cells.

4. Discussion

The analysis of the exposure using qPCR did not find that liquid application of PM caused significantly elevated levels of gene expression related to hypoxia and oxidative stress. Additionally, protein assays for inflammatory cytokines did not find that either liquid application or nebulizer application caused a significant dose response for PM exposure. qPCR analysis of HMOX1 and HIF1A did not reveal any consistent trend in the change of gene expression. No differences between groups were significant, the error bars of nearly all groups included the y-axis value of one (indicates no change in expression), and the plot of the fold change in gene expression shows high levels of variance within treatment groups. ELISA analysis did not reveal any significant differences between groups; however, for IL8 there was an observable positive trend in the bar graph particularly for the liquid exposure.

The results of this project do not support the hypotheses that liquid application of pollutants causes unintended effects including hypoxia, or that mesh nebulizers are an effective alternative exposure method. Cultured primary human airway cells, exposed to PM by liquid application did not exhibit significantly elevated or altered levels of oxidative stress related gene expression. The results of this part of the project would then suggest that liquid application does not cause unintended side effects as we had hypothesized. Further, the analysis of inflammation in cells exposed to PM by nebulizers did not find a significant dose response. Qualitatively the graphs of IL8 response for both liquid application and nebulizer application seem to show a dose response trend, and notably the levels of inflammation in liquid exposure appear to be elevated when compared to nebulized exposure although this difference is not significant. The results of this analysis do not support the use of nebulizers as an alternative exposure method for PM studies. Despite the findings of this project, there are still concerns with liquid application. Additionally, some of the results suggest that with adjustment and refinement there is a possibility that the method of nebulization may have potential as an alternative exposure method.

This project was affected by significant limitations that have important implications for the results and interpretations. First, it is possible that the particle sample selected for the exposures in this experiment was not sufficiently toxic to cause detectable changes in exposed cells. It is known that human generated PM is more dangerous than PM from natural sources [1], so we selected particles from samples of urban PM. However, particles were chosen based primarily on the amount available for use and not comparison with other particles for toxicity. Second, cultured primary cells are well known to have high variability between donors. The cells used for this study were cultured and exposed in two groups of two donors. In addition to the variability present between donors, there was an apparent variation between the two batches of cells, which likely affected the outcomes. Large variation in the responses from donor cells leads to large errors and variability in the results thus making it difficult to observe significant differences between groups. Lastly, the method of nebulizing PM onto cells may be hindered by physical constraints of the system. The mesh nebulizers used in this experiment have a 5 micrometer filter attached to them, and while PM2.5 is under 5 micrometers in diameter, we hypothesize that particles may agglomerate into larger groups of particles that may not be able to make it through the filter on the nebulizer. Hence, delivery of PM to the apical surface of cells may have been significantly impacted and hindered by the small mesh size. Nebulizers are available with larger filters, which could potentially address this issue.

While the results of this project do not support the proposed hypotheses, they do not eliminate concerns with liquid application or rule out nebulizers as a potential alternative exposure method. Inhalation toxicology literature strongly supports the idea that liquid application exposure causes unintended and disruptive repercussions in airway cells, and in the future, more work should be done to confirm the effects of liquid exposure. Additionally, mesh nebulizers and nebulizers in general may have potential as an exposure method. Testing nebulizers with varying physical constraints could reveal a design that is a more biologically relevant method of exposing airway cells to pollutants. Ultimately, evaluating established exposure methods and refining novel methods is important and could progress the field of inhalation toxicology by leading to more biologically relevant models for studying the health effects of airborne substances.

Acknowledgements

I would like to thank all the individuals who helped me throughout this project and to get to this point as a researcher. All the members of the Jaspers, Rebuli, and Alexis labs as well as all members of the Center for Environmental Medicine Asthma and Lung Biology (CEMALB) for making such a welcoming, productive, and enjoyable laboratory environment. Dr. Tim Smythe for providing advice on PM materials for use in this project. Dr. Lauren Eaves, Dr. Rebecca Fry, and all the faculty and students involved in the UNC Gillings School of Public Health QUEST summer program for helping me develop my personal skills as well as a foundation for this project. My peers in the Spring 2025 Biol 692H senior honors thesis stress response group, Ekta Shah, Shubhi Singh, and Yingning Sang for all their feedback and help writing this thesis. Dr. Markus Nevil and Lynn Lerner for their guidance and feedback through the semester of Biol 692H. Charlotte Love and Winny Soerianto for being kind, knowledgeable, and helpful lab mates. Dr. Syed Masood for spending countless hours developing and working on this project with me. Kevin Schichlein for being my mentor for the last year and a half and helping me to learn and develop the skills to be a scientist and complete my own research project. Finally, I would like to thank Dr. Ilona Jaspers for giving me the opportunity and support to work in a lab environment and pursue this research project, and for her invaluable guidance and mentorship.

Author Contributions

Text and figures of this thesis were developed by Benjamin Hawley. All materials were reviewed and edited by Dr. Ilona Jaspers and Kevin Schichlein.

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